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EXPERIMENTAL STUDIES OF YELLOW FEVER IN NORTHERN BRAZIL.

By

HIDEYO NOGUCHI, M.D., HENRY R. MULLER, M.D., OCTAVIO TORRES,
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RIBEIRO dos SANTOS, M.D., GODOFREDO VIANNA, M.D., AND
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EXPERIMENTAL STUDIES OF YELLOW FEVER IN
NORTHERN BRAZIL.

By HIDEYO NOGUCHI, M.D.,

Member of The Rockefeller Institute for Medical Research, New York.

HENRY R. MULLER, M.D.,

*Special Member, Field Staff, International Health Board, The Rockefeller
Foundation.*

OCTAVIO TORRES, M.D., AND FLAVIANO SILVA, M.D.,

*Directoria Geral de Saúde Publica do Estado da Bahia; Faculdade de Medicina
da Bahia.*

HORACIO MARTINS, M.D.,

Bacteriologist, Instituto Oswaldo Cruz da Bahia.

ALVARO RIBEIRO DOS SANTOS, M.D.,

Faculdade de Medicina da Bahia.

GODOFREDO VIANNA, M.D., AND MARIO BIÃO, M.D.,

Serviço de Saneamento e Prophylaxia Rural no Estado da Bahia.

PLATES 1 TO 5.

(Received for publication, June 6, 1924.)

INTRODUCTION.

Yellow fever is known to have existed in classical form in Brazil from the beginning of commercial development until the anti-*Stegomyia* campaigns of Oswaldo Cruz, Ribas, and Lacerda practically eradicated the disease from the country. The striking results of these campaigns are shown in Table I.¹ Most of the important littoral cities, for example, Rio de Janeiro, São Paulo, Santos, and Victoria, have since remained free from yellow fever, but in certain localities, among them

¹ From Boyce, R., Yellow fever and its prevention, New York, 1911, 11.

the states of Ceará and Bahia, the disease has persisted until as recently as 1923. The fluctuation of epidemic yellow fever from year to year in the city of Bahia and the case incidence for each month of the year 1923 are recorded in Text-figs. 1 and 2.²

During the years 1922 and 1923 a small epidemic occurred in Ceará, in Fortaleza, the capital, and also in the interior, where American and British companies were engaged in the construction of dams

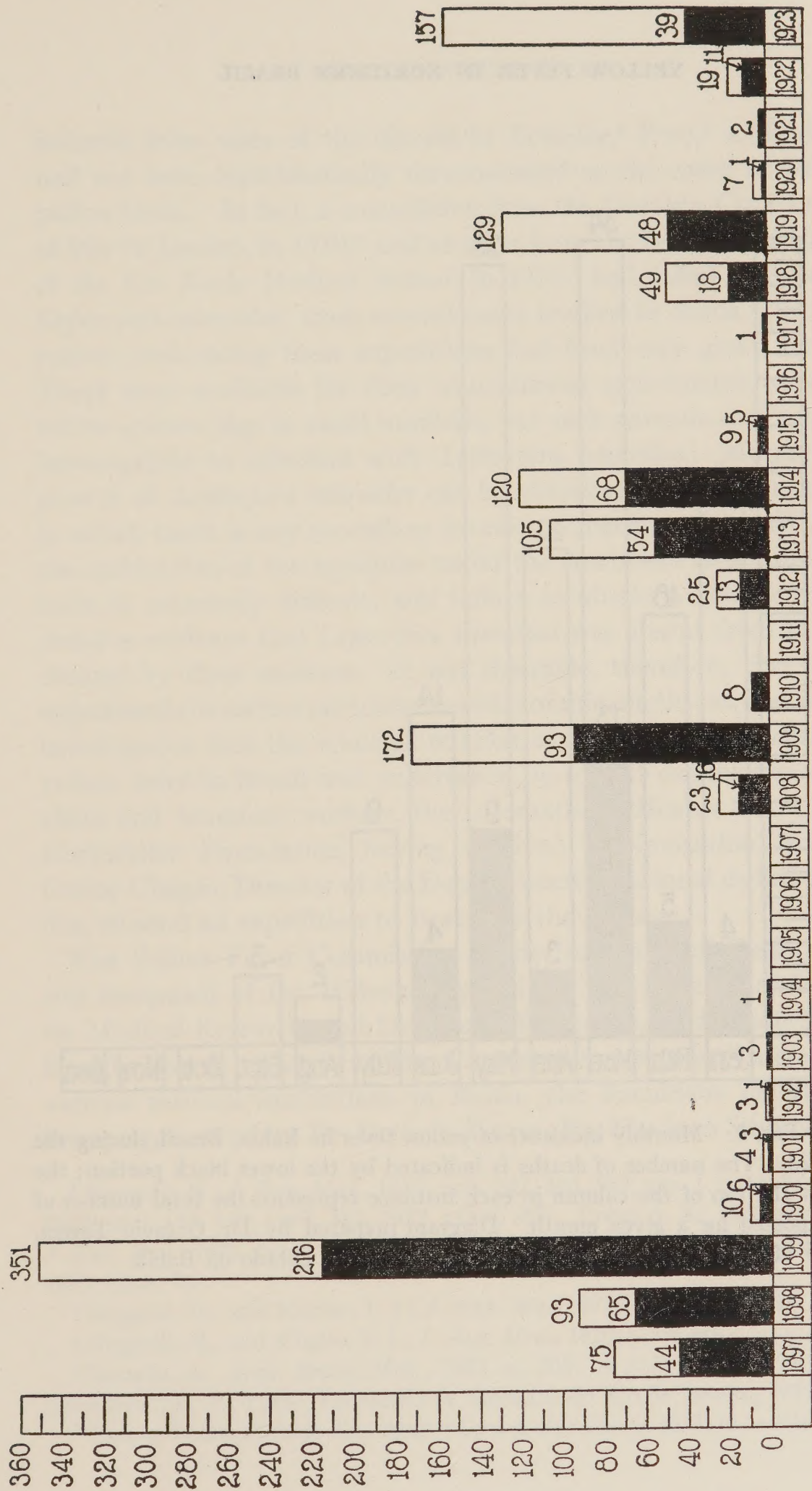
TABLE I.
*Deaths from Yellow Fever in Rio de Janeiro, 1896 to 1904.*¹

Month.	1896	1897	1898	1899	1900	1901	1902	1903	1904
Jan.....	690	38	19	138	57	24	48	184	5
Feb.....	986	65	116	235	49	55	86	219	7
Mar.....	1,433	88	310	258	89	83	223	270	9
Apr.....	557	66	278	101	50	66	218	148	8
May.....	171	36	178	40	21	38	208	44	10
June.....	34	16	82	22	13	22	128	20	4
July.....	24	5	54	17	7	19	85	16	4
Aug.....	16	2	30	7	6	5	54	12	1
Sept.....	4	1	14	14	3	15	38	4	2
Oct.....	19		12	11	7	16	39	4	
Nov.....	12	4	17	19	7	9	36	3	30
Dec.....	28	4	30	35	5	10	121	4	
Total.....	3,974	325	1,140	897	314	362	1,284	928	80

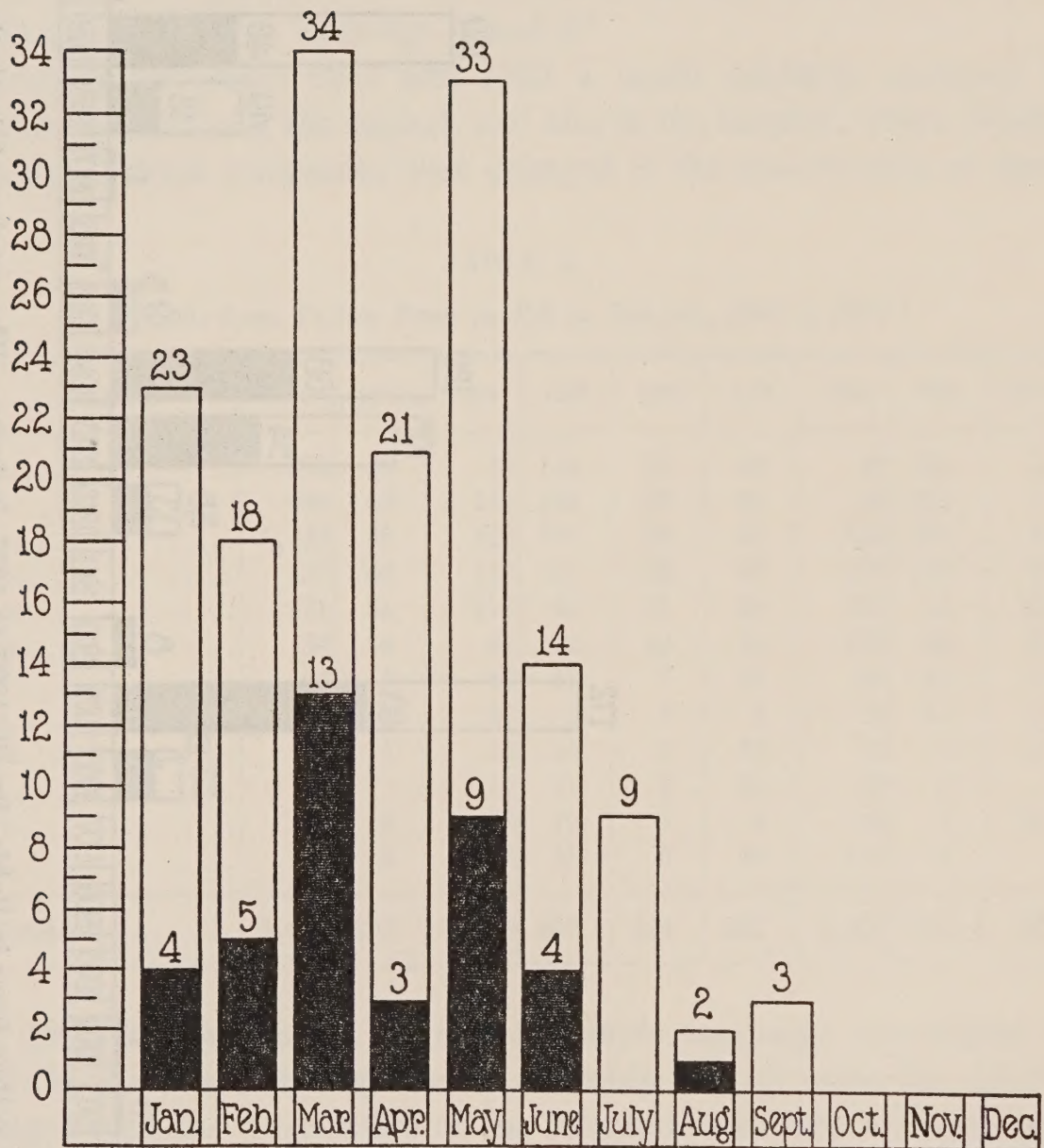
for irrigation purposes. An anti-*Stegomyia* campaign was begun in 1923 by the National Department of Public Health under Dr. Carlos Chagas with the cooperation of the International Health Board, represented by Dr. Joseph H. White, with the result that since September of that year there has been no case of yellow fever in Bahia, Recife, Maceio, Parahyba, Fortaleza, or Pará.

While there was no reason to regard the yellow fever of Brazil as different from yellow fever occurring elsewhere, the microorganism

² Prepared by Dr. Octavio Torres, as director of demographic statistics of the Directoria Geral de Saúde Publica do Estado da Bahia.



TEXT-FIG. 1. Annual incidence of yellow fever in Bahia, Brazil, 1897 to 1923, inclusive. The number of deaths is indicated by the lower black portion; the figure at the top of the column in each instance represents the total number of cases reported for a given year. Diagram prepared by Dr. Octavio Torres, Director of the Estatística Demographo Sanitaria do Estado da Bahia.



TEXT-FIG. 2. Monthly incidence of yellow fever in Bahia, Brazil, during the year 1923. The number of deaths is indicated by the lower black portion; the figure at the top of the column in each instance represents the total number of cases reported for a given month. Diagram prepared by Dr. Octavio Torres, Director of the Estatística Demographo Sanitaria do Estado da Bahia.

isolated from cases of the disease in Ecuador,³ Peru,⁴ and Mexico,⁵ had not been experimentally demonstrated as the cause of Brazilian yellow fever. In fact, a commission from the Oswaldo Cruz Institute of Rio de Janeiro, in 1919,⁶ and another from the Institute of Hygiene of the São Paulo Medical School, in 1921,⁷ had failed to isolate the *Leptospira icteroides* from several cases studied in Bahia. The difficulties confronting these expeditions had been very great, however. There were available for their transmission experiments only adult native guinea pigs in small numbers, yet such animals are practically insusceptible to infection with *Leptospira icteroides*. Moreover, no growth of *Leptospira icteroides* can be obtained in a culture medium in which there is any secondary invasion, fungous or bacterial, hence the cultivation of the organism under the conditions of a tropical climate is extremely difficult, and failure to obtain a culture was not decisive evidence that *Leptospira icteroides* was absent from the cases studied by these workers. It was desirable, therefore, that further experiments be carried out under more favorable conditions, and renewed investigation into the etiologic relation of *Leptospira icteroides* to the yellow fever in Brazil was undertaken by a joint commission of Brazilian and American workers, the International Health Board of The Rockefeller Foundation having received an invitation from Dr. Carlos Chagas, Director of the Departamento Nacional de Saúde Pública, to send an expedition to Brazil for the purpose.

The Yellow Fever Commission of the International Health Board was composed of Dr. Hideyo Noguchi, of The Rockefeller Institute for Medical Research, and Dr. Henry R. Muller, of the International Health Board. The Brazilian Commission comprised members of various medical institutions of Bahia, the Faculdade de Medicina being represented by Dr. Octavio Torres, Dr. Flaviano Silva, and Dr.

³ Noguchi, H., *J. Exp. Med.*, 1919, xxix, 547, 565, 585; xxx, 1, 9, 13, 87, 95, 401; 1920, xxxi, 135, 159; xxxii, 381; 1922, xxxvi, 357. Cohn, A. E., and Noguchi, H., *J. Exp. Med.*, 1921, xxxiii, 683. Noguchi, H., and Pareja, W., *J. Am. Med. Assn.*, 1921, lxxvi, 96.

⁴ Noguchi, H., and Kligler, I. J., *J. Exp. Med.*, 1921, xxxiii, 239, 253.

⁵ Noguchi, H., and Kligler, I. J., *J. Exp. Med.*, 1920, xxxii, 601.

⁶ Barreto, A., *Arch. Brasil. Med.*, 1921, xi, 205; *A Folha Med.*, 1920, i, 152. Cavalcanti, E. P., These, Faculdade de Medicina do Rio de Janeiro, 1921.

⁷ Borges Vieira, F., *Bol. Soc. Med. e Cirurg. São Paulo*, 1921, iv, series 2, 137.

Alvaro Ribeiro dos Santos, the Instituto Oswaldo Cruz by Dr. Horacio Martins, and the Serviço de Saneamento e Prophylaxia Rural by Dr. Godofredo Vianna and Dr. Mario Bião. Drs. Octavio Torres and Flaviano Silva also represented the Directoria Geral de Saúde Publica da Bahia.

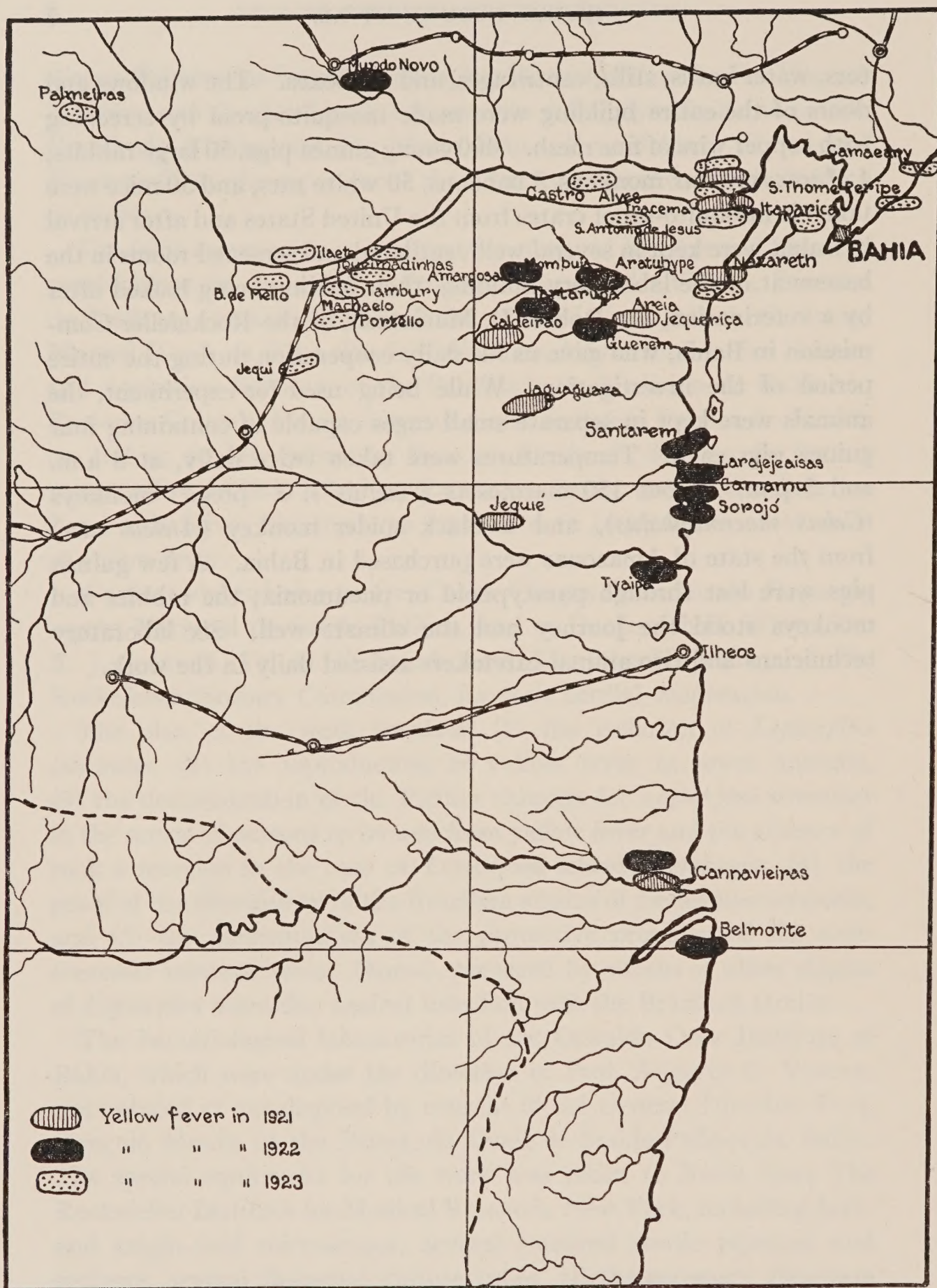
The joint commission is indebted for constant interest and frequent participation in the work to Professor Augusto C. Vianna, Director do Instituto Oswaldo Cruz da Bahia; to Professors João A. G. Fróes, Manoel Pirajá da Silva, Prado Valladares, Mario Andréa dos Santos, and Agrippino Barbosa, of the Faculdade de Medicina da Bahia; to Professor Gonçalo Moniz, Director Geral de Saúde Publica do Estado da Bahia; to Dr. Sebastião Barroso, Chefe do Serviço de Saneamento e Prophylaxia Rural no Estado da Bahia, and to Dr. Americo Pereira da Silva, who made an expedition to Jequy in search of cases of yellow fever.

We are indebted also to Dr. Carlos Chagas, Director do Departamento Nacional de Saúde Publica, and to Dr. Joseph H. White, Dr. E. J. Scannell, Dr. G. K. Strode, and Dr. John H. Janney, of the Rockefeller Sanitary Commission, for their cordial cooperation.

The plan of the work involved (1) the isolation of *Leptospira icteroides*, (2) the reproduction of yellow fever in lower animals, (3) the demonstration of the Pfeiffer reaction for *Leptospira icteroides* in the serum of persons recovered from yellow fever and the absence of such a reaction in the case of *Leptospira icterohæmorrhagiæ*, (4) the proof of the filterability of the Brazilian strains of *Leptospira icteroides*, and (5) the determination of the protective property of the anti-*icteroides* immune serum (horse), prepared by means of other strains of *Leptospira icteroides*, against infection with the Brazilian strains.

The bacteriological laboratories of the Oswaldo Cruz Institute of Bahia, which were under the direction of Prof. Augusto C. Vianna, were placed at our disposal by consent of the General Director, Prof. Gonçalo Moniz, of the Directoria Geral de Saúde Publica da Bahia. The special equipment for the work was taken to Bahia from The Rockefeller Institute for Medical Research, New York, including dark- and bright-field microscopes, several hundred sterile pipettes and syringes, several thousand culture tubes, all the necessary glassware and stains, filters, apparatus for hot air and steam sterilization, incuba-

tors, water baths, stills, centrifuges, and ice boxes. The windows and doors of the entire building were made mosquito-proof by screening with copper wire of fine mesh. 400 young guinea pigs, 50 large rabbits, 4 *Macacus rhesus* monkeys, 3 baboons, 50 white rats, and 50 mice were taken in mosquito-proof crates from the United States and after arrival in Bahia were kept in several well ventilated and screened rooms in the basement of the laboratory building, their feeding being looked after by a veterinarian, Dr. Robert D. Macintosh, of the Rockefeller Commission in Bahia, who gave us his daily cooperation during the entire period of the investigation. While being used for experiment, the animals were kept in separate small cages capable of containing four guinea pigs each. Temperatures were taken twice daily, at 8 a.m. and 5 p.m. About 150 marmosets ("saguis"), 3 "prego" monkeys (*Cebus macrocephalus*), and a black spider monkey (*Ateles ater*) from the state of Amazonas were purchased in Bahia. A few guinea pigs were lost through paratyphoid or pneumonia; the rabbits and monkeys stood the journey and the climate well. Six laboratory technicians and two animal caretakers assisted daily in the work.



TEXT-FIG. 3. Map of the State of Bahia, showing the gradually increasing occurrence of yellow fever in several interior towns during the years 1921, 1922, and 1923.

ISOLATION OF LEPTOSPIRA ICTEROIDES FROM CASES OF YELLOW FEVER IN THE INTERIOR OF BAHIA.

Three cases of yellow fever had occurred in Bahia in September, 1923, but since that time the city had remained free from the disease, owing to the effective anti-*Stegomyia* campaign conducted by the joint staff of the Federal Prophylaxia Rural and the local branch of the Rockefeller Sanitary Commission under Dr. Scannell. Hence no cases were available for transmission experiments when we began our work in November. About the middle of December, however, Dr. Sebastião Barroso, chief of the Federal Prophylaxia Rural of the state, received information that in Villa Bella das Palmeiras (Text-fig. 3), an interior city of 3,000 inhabitants, a disease believed by a local physician to be yellow fever had been prevalent for several weeks. There was no railroad connection between Bahia and Palmeiras; the journey required 1 day by steamer, 1 by train, and 4 on horseback. The distance and the character of the journey made the transportation of experimental animals out of the question, but there was a possibility of obtaining cultures by inoculation of suitable culture medium transported in a water-cooled box. Dr. Godofredo Vianna and Dr. Mario Bião were selected by Dr. Barroso from his staff to undertake the expedition to Palmeiras, establish the diagnosis, and obtain cultures in the event that the disease proved to be yellow fever. Their equipment included clinical thermometers, Esbach reagent, microscope, slides, and Giemsa stain for examination for malarial parasites,⁸ autopsy set, sterile syringes, disinfectants, alcohol lamps, and finally 80 hermetically sealed tubes of culture medium, which had been prepared at The Rockefeller Institute and transported uninterruptedly under refrigeration (4°C.). During the return journey from Palmeiras the inoculated tubes were partly submerged in a case containing water and covered with green leaves to protect them from the excessive

⁸ In order to check the results of blood examination, several extra smears were made in each instance, fixed in methyl alcohol, and brought back to be stained and examined in the laboratory in Bahia.

heat of the daytime; otherwise the cultures would no doubt have been dead before reaching Bahia.

The medium used for this expedition differed from that employed for routine subcultures of *Leptospira icteroides* in that it contained no hemoglobin and was prepared with distilled water as the diluent instead of isotonic saline or Ringer solution. The omission of the hemoglobin renders the reaction of the medium practically stable to ordinary temperatures, at which hemoglobin rapidly undergoes modification, while the replacement of the isotonic diluent by distilled water results in liberation from the patient's blood of sufficient hemoglobin for the growth of *Leptospira icteroides*. The formula of this medium is as follows:

Fresh rabbit serum.....	100 parts
Distilled water.....	800 “
2 per cent nutrient agar (pH 7.5).....	100 “

The mixture of rabbit serum and distilled water is kept at a temperature of 50°C., and the melted agar, cooled to that temperature, is added quickly. The whole is rapidly mixed and distributed immediately, before the agar flocculates, into culture tubes 20 × 200 mm. (so called Noguchi tubes), 10 cc. to each. The tubes are put into the refrigerator (4°C.) to hasten solidification into an almost transparent semigelatinous homogeneous mass. If the agar flocculates and does not form a homogeneous mixture with the other ingredients, the medium is unsuitable for cultivation, hence the difficulty of preparing culture medium in a tropical climate, unless a good refrigerator is available.

For subcultures the following formula is recommended:

0.9 per cent NaCl.....	800 parts
Fresh rabbit serum.....	100 “
2 per cent nutrient agar (pH 7.5).....	100 “
Rabbit hemoglobin (made by laking 1 part of the defibrinated blood in 3 parts of distilled water).....	10-20 “

This medium undergoes rapid modification at ordinary temperatures and should be kept in the ice box until used.

These seemingly trifling precautions with regard to the preparation of the culture medium are of great practical importance, and ignorance

of their significance might have played some part in the failure of earlier investigators to isolate the leptospira.

The diagnosis of yellow fever in Palmeiras was confirmed, and cultures were made from the blood of one fatal and four non-fatal cases and brought back by Dr. Vianna on January 4, 1924. In each instance six tubes of medium were inoculated with quantities of blood in gradually decreasing amounts from 2 cc. to a few drops, this procedure being followed in order to avoid excess of laked blood in some tubes and consequent interference from changes in reaction due to gradual aging of hemoglobin.

The tubes, thirty in all, were divided among Drs. Octavio Torres, Flaviano Silva, Alvaro Ribeiro dos Santos, Horacio Martins, Godofredo Vianna, and Muller for immediate examination under the dark-field microscope. There had been a mould or bacterial invasion in some tubes, but the majority were free from contamination—an unusual accomplishment in a tropical region. In the course of a rather rapid examination, Dr. Vianna found a rich growth of the leptospira in the fifth tube from Case 3. None of the other tubes revealed any leptospiras at that time, but examinations were continued for several successive days, and another culture was subsequently found by Dr. Horacio Martins in the second tube from Case 5. The leptospira was therefore isolated in two of the five cases similarly studied.

The failure of the organisms to grow in the other tubes was not peculiar to the human material, for in the case of some of the subsequent animal experiments with the Palmeiras strains only one or two of a dozen tubes inoculated with the infective blood of experimental animals yielded a culture, and in some instances cultures were altogether negative.

Case 1.—J. N. d. S., male, age 18 years, white; resident of Palmeiras. First seen on Dec. 27, 1923, 1st day of illness. Cephalalgia, rachialgia, pains in lower extremities. Temperature 101.3°F., pulse 100. No albumin in urine. Blood taken for cultivation. Blood smears negative for malarial parasites. Dec. 28. Headache less intense, general pains. Temperature a.m. 102°, p.m. 104°; pulse a.m. 110, p.m. 100. Nausea; volume of urine 600 cc., traces of albumin. Dec. 29. Epigastric pain, nausea, depression, insomnia. Volume of urine 500 cc., albumin 0.3 gm. Temperature a.m. 103°, p.m. 102°; pulse a.m. 90, p.m. 110. Dec. 30. Asthenia, icterus. Temperature 99°, pulse 80. Albumin 0.3 gm.

Case 2.—J. G., male, age 10 years, white; resident of Palmeiras. Onset with pains in lower extremities and throughout body, intense headache, slight chills and fever. First seen Dec. 23, 2nd day of illness. Pains, backache as on previous day, headache less intense. Face puffy, tongue coated; photophobia. Palpation revealed tenderness of left lobe of liver. Temperature (axilla) 102.7°F., pulse 140. Total volume urine 900 cc. Dec. 24. Headache and leg pains persist; patient says he is better. Temperature (axilla) a.m. 100.7°, p.m. 102°; pulse 120. Volume of urine 900 cc., albumin 1.5 gm. Blood withdrawn for cultivation. Blood smears negative for malarial parasites. Dec. 25. Asthenia. Temperature (axilla) a.m. 99.6°, pulse 100; same in p.m. Volume of urine 400 cc., albumin 1 gm. Dec. 26. Restlessness, epistaxis. Temperature (axilla) 99.8°, pulse 110. Dec. 27. Black vomit, epistaxis, asthenia, very marked icterus. Temperature (axilla) 97.7°, pulse 116. Melena. Dec. 28. Complete anuria. Death at 2 a.m. Body completely yellow (color of sulfur).

Case 3.—A. V. B., female, age 23 years, mulatto, single; resident of Palmeiras. Onset Dec. 25, 1923, with severe headache, vertigo, epigastric pain, bilious vomiting, pains in legs, backache. Temperature 103.1°F., pulse 110. Blood withdrawn for cultivation. Blood smears negative for malarial parasites. 400 cc. of urine passed in 16 hours, albumin 0.5 gm. Dec. 26. Headache, backache, vomiting. Temperature a.m. 102°, p.m. 100.4°; pulse a.m. 110, p.m. 96. Total volume of urine 400 cc., albumin 1.5 gm. Dec. 27. Headache less severe. Temperature a.m. and p.m. 97.7°, pulse 90. Asthenia. Albumin 0.5 gm. Dec. 28. Temperature 97.7°, pulse 88. Jaundice. Albumin 0.5 gm.

Case 4.—J. V., male, age 18 years, white, single; resident of Palmeiras. Onset with severe headache and general pains, particularly severe in the legs. High fever afternoon of same day; nausea but no vomiting. First seen on 2nd day, Dec. 23. Headache severe, pains in legs, backache, photophobia. Temperature (axilla) a.m. 103.1°F., p.m. 103.6°; pulse 120. Total volume of urine 800 cc., albumin 1 gm. Alimentary and bilious vomiting. Dec. 24. Continued headache, gingivitis, bilious vomiting. Temperature (axilla) a.m. 102.5°, p.m. 103.1°. Total volume of urine 600 cc., albumin 2.5 gm. Dec. 25. Patient feels better; no headache, volume of urine increased. Temperature (axilla) a.m. 99.1°, pulse 85. Albumin 0.5 gm. Blood withdrawn for cultivation. Blood smears negative for malarial parasites. Dec. 26. Asthenia, epigastric pain, icterus of the conjunctivæ. Temperature (axilla) 96.8°, pulse 98. Albumin 1 gm. Dec. 27. Asthenia. Temperature and pulse normal; albumin 0.5 gm. Dec. 28. Convalescent. Temperature normal, pulse 52. Asthenia, general icterus.

Case 5.—J. B. d. S., male, age 25 years, mulatto, single; resident of Palmeiras. Onset Dec. 25 at 1 p.m., with severe headache, backache, pains in legs, epigastric pain. First seen Dec. 26, 9 a.m. Intense headache, backache, epigastrium tender to palpation; distention from gas in iliac fossa; frequent vomiting of bile-stained fluid. Volume of urine 1,000 cc. Temperature (axilla) a.m. 102°F., p.m. 101.8°; pulse a.m. 110, p.m. 106. Blood withdrawn for cultivation. Blood smears negative for malarial parasites. Dec. 27. Headache less severe; epistaxis; epigastric

pain. Temperature a.m. 100° , pulse 88. Trace of albumin in urine. Volume 1,000 cc. Dec. 28. General malaise; epistaxis. Volume of urine 950 cc. Temperature a.m. 97.1° , p.m. 100° ; pulse a.m. 72, p.m. 84. Dec. 29. Asthenia. Temperature 96° , pulse 50. Albumin 0.5 gm.

Dr. Bião remained in Palmeiras 26 days after Dr. Vianna's return to Bahia and was able to make cultures from four more cases. These tubes were kept in Palmeiras for a much longer time than the first series, and when they finally reached the Instituto Oswaldo Cruz, a large percentage of them was contaminated. The leptospira was not detected in any instance, notwithstanding repeated search during several consecutive days, a result which was expected in view of the prolonged exposure to unfavorable conditions. That the cases of this series were genuine yellow fever was subsequently demonstrated by the positive Pfeiffer reaction obtained with the sera from these patients when tested with the Palmeiras strains as well as with strains from Peru and Mexico.

Case 6.—M. d. A., female, age 18 years, white, single; resident of Palmeiras. Onset Jan. 19, 1924, with headache, chills, fever, pains in legs, backache. First seen afternoon of same day. Temperature 104°F. , pulse 120. Volume of urine normal. Jan. 20. Continued headache, backache. Temperature 101.2° , pulse 80. Volume of urine 1,000 cc., albumin 1 gm. Blood withdrawn for cultivation. Jan. 21. Patient feels well. Temperature a.m. 101.8° , p.m. 99° , pulse a.m. 85, p.m. 70. Volume of urine 800 cc., traces of albumin. Jan. 22. Convalescent.

Case 7.—B. d. A., female, age 22 years, white; resident of Palmeiras. Onset Jan. 10, 1924, with severe headache, pains in legs, backache. Seen on afternoon of same day: face puffy, tongue coated, with red edges, photophobia, frequent alimentary vomiting. Temperature (axilla) 104° , pulse 120. Jan. 11. Continued headache, backache; left lobe of liver tender to palpation; epigastric pain, bilious vomiting. Temperature a.m. 101.5° , p.m. 103° ; pulse a.m. and p.m. 120. Patient eliminated no urine for 12 hours, hence examination for albumin could not be made. Jan. 12. Frequent bilious vomiting; continued headache, restlessness. Temperature (axilla), a.m. 101° , p.m. 104° ; pulse a.m. 100, p.m. 120. Epigastric distress. Volume of urine 500 cc., traces of albumin. Blood withdrawn for cultivation. Jan. 13. Continued bilious vomiting and epigastric pain; slight abdominal tympany; icterus of conjunctivæ. Temperature 104° , pulse 120. Volume of urine 500 cc., albumin 0.5 gm. Jan. 15. Icterus, atony. Temperature a.m. 102° , p.m. 103° ; pulse a.m. 90, p.m. 100. Albumin 1 gm. Jan. 16. Icterus, atony. Temperature a.m. 101° , p.m. 100° ; pulse a.m. 80, p.m. 70. Traces of albumin in urine. Jan. 17. Icterus, asthenia. Temperature 98° , pulse 70. No albumin in urine. Jan. 18. Asthenia. Temperature and pulse normal.

Case 8.—J. F. d. A., female, age 55 years, mulatto, married; resident of Palmeiras. Onset during the night Jan. 14, 1924, with headache, pains in legs, neck, and back. Seen on afternoon of 2nd day: continued pains; frequent alimentary vomiting. Temperature 104°F., pulse 120. Patient reports normal volume of urine. Jan. 16. Headache persists; epigastric distress; alimentary and bilious vomiting. Temperature a.m. 101.2°, p.m. 101.5°; pulse a.m. and p.m. 80. Blood withdrawn for cultivation. Albumin 1 gm. Jan. 17. Asthenia; continued epigastric distress. Temperature 99°, pulse 70. Traces of albumin in urine.

Case 9.—J. J. M., male, age 20 years, negro, single; resident of Palmeiras. Onset Jan. 8, 1924, with intense headache, chills and fever, pains in legs, backache, alimentary vomiting. Temperature (axilla) a.m. 103°F., pulse 100. Volume of urine 1,000 cc. In the afternoon complained of severe vesical pain and epigastric distress. Temperature 103°, pulse 100. No albumin in urine. Jan. 9. Headache, backache, alimentary vomiting. Temperature a.m. 100°, pulse 70; same in p.m. Volume of urine 800 cc., no albumin. Jan. 10. Asthenia, slight icterus of conjunctivæ. Temperature 97°, pulse 70. Volume of urine 600 cc., albumin 0.5 gm. Blood withdrawn for cultivation. Jan. 11. Asthenia. Temperature and pulse normal; traces of albumin in the urine.

The yellow fever among the native population, as these case histories show, has been rather mild, compared with the severe form of the disease from which a foreigner usually suffers.

PATHOGENICITY AND BIOLOGICAL PROPERTIES OF THE BRAZILIAN STRAINS.

To determine the specific pathogenicity of the strains of *Leptospira icteroides* isolated from cases of yellow fever in Palmeiras, guinea pigs were used which had been reared in a temperate climate, and which weighed 200 to 280 gm. Marmosets were available in the markets of Bahia, and since the Ecuadorean species of this monkey had proved susceptible to *Leptospira icteroides*,⁹ about 150 Brazilian marmosets weighing 100 to 150 gm. were purchased. They proved, however, not only relatively insusceptible to *Leptospira icteroides*, but non-resistant to spontaneous bacterial infections under the conditions of captivity, hence they were used only in the earlier transmission experiments. Reproduction of yellow fever was obtained in a striking way, however, in two native monkeys, and later in two young dogs.

As already stated, two strains of *Leptospira icteroides* had been obtained in culture from two of nine cases of yellow fever occurring in Palmeiras. These have been designated respectively as Strain 3 and Strain 5 (Figs. 1 and 2).

I. Experiments with Strain 3.

Tube 5 of the series of six tubes inoculated with the blood of Palmeiras Case 3 had shown a fairly rich growth of *Leptospira icteroides* on dark-field examination immediately after Dr. Vianna's arrival from Palmeiras. It was inoculated into guinea pigs and marmosets the same day (January 4, 1924), in order to avoid possibility of loss by secondary contamination. The culture was suspended in enough isotonic saline solution to furnish 1 cc. for each animal. The inoculations were made intraperitoneally.

Inoculation of Guinea Pigs.

Guinea Pig I, Cage 52. Inoculated Jan. 4, 1924. Moderately high fever was observed after 4½ days incubation and had persisted for 48 hours when the animal

⁹ Noguchi, H., *J. Exp. Med.*, 1919, xxix, 585.

was sacrificed and the heart blood used for transfers. No leptospiras were found in the blood or organ suspensions.

Autopsy.—No jaundice, slight hemorrhage; organs not typically altered. Probably beginning *icteroides* infection.

Transfers from Guinea Pig I, Cage 52.

Guinea Pig A, Cage 73. Inoculated Jan. 11, 1924. Fever (105°F.) appeared after 4 days incubation and persisted 48 hours, after which slight jaundice was noticed. Blood obtained by heart puncture showed no leptospira on dark-field examination, but growth was obtained subsequently in one of eleven tubes of culture medium. The animal died shortly after heart puncture. It showed typical lesions at autopsy.

Guinea Pig B, Cage 73. Duplicate of the foregoing. Moderate fever (104.4°F.) after 6 days, lasting for 11 days. Observations were discontinued at this time. No jaundice or hemorrhages. Diagnosis: Mild paratyphoid infection.

Marmoset, Cage 74. Duplicate of the foregoing. Fever 104°F. 48 hours after inoculation; died 2 days later after showing subnormal temperature. Autopsy showed no signs of *icteroides* infection.

Guinea Pig II, Cage 52. Inoculated Jan. 4, 1924. After an incubation period of 6 days, the temperature rose to 104.4°F. Heart blood was withdrawn for transfer to three guinea pigs and a marmoset. No leptospira found in blood or cultures. The animal was found dead 3 days later.

Autopsy.—Generalized jaundice, subcutaneous hemorrhages, petechial hemorrhages in the mucosa of stomach and intestines; lungs showed pneumonia; spleen enlarged, liver pale yellowish, kidneys congested. No leptospira in blood or organ suspensions. Diagnosis: *Icteroides* infection complicated with paratyphoid and pneumonia.

Transfers from Guinea Pig II, Cage 52.

Guinea Pig A, Cage 76. Inoculated Jan. 10, 1924. Fever 104.6–104.8°F. after 5 days incubation. Blood obtained by heart puncture for cultivation and dark-field examination. Animal died 48 hours later.

Autopsy.—Jaundice, hemorrhages, and nephritis; spleen enlarged; liver paler than normal. Dark-field examination showed no leptospira, but one of the nine tubes inoculated with heart blood yielded a culture. Diagnosis: *Icteroides* infection complicated with paratyphoid.

Guinea Pig B, Cage 76. Duplicate of the foregoing. Fever began after 4 days incubation; the animal was sacrificed 48 hours later.

Autopsy.—Mild jaundice, typical hemorrhages into lungs and gastrointestinal tract, liver slightly paler than normal. Dark-field examination revealed a few leptospiras in the blood. Subsequent transfers established a virulent strain in guinea pigs.

Guinea Pig C, Cage 76. Duplicate of the foregoing. Fever began 4 days after inoculation and persisted 2 days. Blood withdrawn by heart puncture. Animal died during the night.

Autopsy.—No jaundice, but characteristic hemorrhages. Pneumonia. Spleen enlarged. Diagnosis: Beginning *icteroides* infection complicated by paratyphoid and pneumonia.

Marmoset, Cage 77. Duplicate of the foregoing. Fever began 3 days after inoculation, and the animal died 40 hours later.

Autopsy.—Pneumonia; no signs of *icteroides* infection.

Marmosets, Cages 75 and 76. Inoculated in the same way as the foregoing, died of pneumonia in 4 and 5 days, respectively.

Guinea Pig III, Cage 52. Inoculated Jan. 4, 1924. After 5 days incubation, the temperature rose to 104–104.2°F., but subsided within 36 hours. No attempt was made to transfer heart blood during the febrile stage, since transfers had been made in the case of two other animals showing similar febrile reaction. The animal had no other symptoms but perhaps there was a mild *icteroides* infection.

Guinea Pig IV, Cage 52. Inoculated Jan. 4, 1924. Except for a rise of temperature to 104.6–105°F. 4 days after inoculation, the animal showed nothing of interest. Heart puncture at the febrile stage and subsequent passage to guinea pigs might have demonstrated the *icteroides* infection.

Guinea Pig V, Cage 53. Inoculated Jan. 4, 1924. Moderate fever for 36 hours after 4½ days incubation; no other symptoms. Perhaps a mild *icteroides* infection.

Guinea Pig VI, Cage 53. Inoculated Jan. 4, 1924. Had no fever at any time but died 4 days after inoculation.

Autopsy.—Paratyphoid infection; no *icteroides* lesions.

Guinea Pig VII, Cage 53. Inoculated Jan. 4, 1924. The highest temperature, 5 days after inoculation, was 103.8°F.; persisted 1 day. 3 days later the animal was jaundiced and showed subnormal temperature (100°F.). Died.

Autopsy.—Jaundice, petechial hemorrhages into lungs; stomach contained dark altered blood (black vomit); liver fatty, showed some necrotic areas; spleen enlarged. Diagnosis: *Icteroides* infection complicated with paratyphoid invasion of the spleen.

Guinea Pig VIII, Cage 53. Inoculated Jan. 4, 1924. After 5 days incubation, temperature was 104.2–104°F. for 1 day. Death 4½ days later.

Autopsy.—Mixed infection, *icteroides* and paratyphoid. The animal appeared to have been recovering from the *icteroides* infection when it succumbed to paratyphoid.

Inoculation of Marmosets.

Marmoset I, Cage 54. Inoculated Jan. 4, 1924. No high fever at any time; died 6 days after inoculation.

Autopsy.—No characteristic *icteroides* lesions. Trace of jaundice in scleræ. A few immobile leptospiras were found in the kidney suspension, none in blood or liver. Death probably due to some other cause than the *icteroides* infection.

Marmoset II, Cage 54. Inoculated Jan. 4, 1924. Died 4 days after inoculation; no fever at any time. Autopsy negative.

Marmoset III, Cage 55. Inoculated Jan. 4, 1924, with Case 3 culture. Definite sharp rise of temperature to 104.2–104°F. after 5 days incubation; persisted 1 day only. Animal died 48 hours later, after showing general jaundice.

Autopsy.—Hemorrhagic points in lungs; gastric mucosa congested; intestines free; spleen normal; fatty degeneration of liver; kidneys pale. No leptospiras demonstrated.

Marmoset IV, Cage 55. Inoculated Jan. 4, 1924. Temperature 104°F. for 1 day, 5 days after inoculation; died 72 hours later, temperature subnormal (96°) just before death.

Autopsy.—A few pin-point hemorrhages in lungs; no other changes characteristic of infection with *Leptospira icteroides*. Transfer of kidney suspension to two guinea pigs produced only a suggestive febrile reaction; cultures made with the heart blood were negative.

The experiments with marmosets were in general highly unsatisfactory, only one (Marmoset III, Cage 55) developing the typical *icteroides* lesions. Many uninoculated animals died without showing any lesions which would explain death.

Attempts were made, nevertheless, to transmit infection to guinea pigs and other marmosets by means of heart blood or kidney suspensions from some of the marmosets of the foregoing protocols.

Three young guinea pigs were inoculated on Jan. 11, 1924, with the suspension of heart blood from Marmoset I, Cage 54. None developed the *icteroides* infection. On the other hand, one of the four guinea pigs inoculated with the kidney suspension (containing a few immobile leptospiras) of the same marmoset showed typical *icteroides* lesions at death (8 days). Of the other three, one died of paratyphoid infection within 6 days, the other two had irregular fever for a few days but never showed definite symptoms—they may have had a mild *icteroides* infection. A marmoset inoculated at the same time with the kidney suspension died of other causes in 7 days; autopsy was negative.

The heart blood of Marmoset IV, Cage 55 was inoculated into a guinea pig and the kidney suspension into two marmosets. All died of pneumonia and showed no *icteroides* lesions at autopsy.

II. Experiments with Strain 5.

One of the guinea pigs (No. I, Cage 63) inoculated on January 4, 1924, with a mixture of three uncontaminated culture tubes from Case 5 (negative for leptospira by dark-field examination) had a temperature of 104–104.6°F. 5 days after inoculation; fever persisted for 4 days, a period rather longer than the usual in *icteroides* infection, and the animal finally recovered without showing definite jaundice. Three other guinea pigs similarly inoculated died overnight of peritonitis, hence it could not be determined whether or not the pooled culture used for inoculation had contained *Leptospira icteroides*, though the reaction in the first guinea pig suggested a mild infection. Continued dark-field search of the culture tubes, however, finally resulted in the finding by Dr. Horacio Martins of a rich growth in the second tube of the Case 5 set.

The pathogenicity tests of this culture were made chiefly in guinea pigs, the preceding experiments having shown the fragility to handling and comparative insusceptibility to *icteroides* infection of the marmosets. The amount of the culture used was 0.5 cc. The inoculations were made intraperitoneally.

Guinea Pig I, Cage 112. Inoculated Jan. 17, 1924. Fever developed 6 days after inoculation and had lasted 72 hours when heart puncture was made to obtain blood for transfer. The animal died as a result of the operation.

Autopsy.—Slight jaundice, hemorrhages into stomach and intestines; petechial hemorrhages in lungs. Dark-field examination negative for leptospira.

Transfers from Guinea Pig I, Cage 112.

Guinea Pig A, Cage 118. Inoculated Jan. 27, 1924. Fever appeared 4 days later and had continued 72 hours when heart puncture was made. Animal died.

Autopsy.—Jaundice of citrated blood; characteristic hemorrhages into stomach, intestines, and lungs; spleen normal, liver pale. Dark-field examination and cultures negative for the leptospira.

Guinea Pig B, Cage 118. Duplicate of the foregoing. High fever developed after 4 days incubation and lasted 48 hours. Jaundice appeared a day later. Blood obtained by heart puncture. Blood plasma jaundiced, but contained no leptospira; cultures remained sterile.

Autopsy.—Marked jaundice, hemorrhages into stomach, intestines, and lungs; spleen normal, liver pale.

Guinea Pig II, Cage 112. Inoculated Jan. 17, 1924. Temperature rose after 5 days incubation and remained high for 72 hours. 2 days later jaundice was noted, and blood was withdrawn by heart puncture for transfer and culture. Animal died overnight.

Autopsy.—Typical *icteroides* lesions, complicated with paratyphoid. No leptospira found in blood or organs. Culture tubes remained negative. Transfer was made to Guinea Pig A, Cage 119 (Jan. 27, 1924), which died of pneumonia within 7 days.

Guinea Pig III, Cage 112. Inoculated Jan. 17, 1924. Showed no febrile reaction and died in 7 days of pneumonia.

Autopsy.—Mild form of *icteroides* infection, probably passing away when pneumonia intervened.

The results established the characteristic pathogenicity for guinea pigs of the leptospira strains obtained from Palmeiras cases, the essential features of the lesions being hemorrhages into the lungs and gastrointestinal mucosa, nephritis, and fatty degeneration of the liver. Jaundice is always present in definite infections; in the milder forms of the disease a febrile reaction, beginning after a period of 4 to 6 days and persisting 1 day or longer, may be the only objective symptom, but positive results may be secured by transferring to other guinea pigs blood or suspensions of organs taken during the febrile stage. The demonstration of the leptospira in such materials can rarely be made, and even in the case of very marked infection, the leptospira is seldom found, while blood or organs taken after the fall of fever are usually non-infective. The success of cultivation is also very variable and never readily accomplished. Secondary infections (paratyphoid and pneumonia) complicated our work in guinea pigs and marmosets considerably. The enlargement of the spleen noted in many instances was due to the paratyphoid complication.

The duration of the febrile stage was about 72 hours in the case of Strain 5, as compared with 24 to 48 hours with Strain 3. In view of the longer febrile period in the animals infected with the Strain 5 culture, it now appears that the first guinea pig inoculated on January 4 with the pooled contents of Case 5 tubes had a mild infection and recovered. The duration of fever in this animal (96 hours) which raised doubt as to the nature of the infection, was only a day longer than that which proved to be the average for this strain. No doubt

the strain could have been recovered from this animal by timely transfer of blood or organ suspension at the beginning of fever.

Enhancement of Virulence by Passage.

The initial cultures of the Palmeiras strains showed very low virulence for guinea pigs and were even less pathogenic for marmosets. A 1:10 dilution of the rich culture of Strain 3 produced a fatal infection in only three of eight guinea pigs, and there was almost always a secondary infection with the paratyphoid bacillus in fatal instances.

TABLE II.

Determination of Minimum Lethal Dose of Palmeiras Strains.

Guinea pig No.	Amount of blood-kidney suspension injected.	Result.	Remarks.
	cc.		
104 A	1	Typical infection; died in 6 days.	Culture obtained in one of the nine tubes inoculated with blood.
104 B	0.1	“ “ “ “ 6 “	Leptospira found in blood.
105 A	0.01	Lost through secondary infection (pneumonia).	
105 B	0.01	Typical infection; died in 7 days.	Died following heart puncture; early lesions present.
106 A	0.001	Definite infection; died in 7 days following heart puncture.	
106 B	0.001	Definite infection; died in 8 days.	
106 C	0.0001	Lost through secondary infection (pneumonia).	

A second passage, however, yielded a greater proportion of fatal infections.

To determine the minimum lethal dose of the passage strains, two guinea pigs (No. A, Cage 73, and No. B, Cage 76), representing the third passage of both strains, were killed during the febrile stage on January 15 and 16, 1924, and the citrated blood and kidney suspensions were mixed for titration. Dark-field examination was negative on routine search, but growth was obtained in one of eleven tubes inoculated with blood from Guinea Pig A, hence there must have been a small number of leptospiras present. The inoculations were made

intraperitoneally. The results obtained in guinea pigs¹⁰ are recorded in Table II.

The difference in virulence between the suspension used and the original culture from the patient is very striking. The number of leptospiras present in the original culture suspension used for inoculation must have been at least 10,000,000 per cc., while the same volume of the suspension used for titration could not have contained more than a few thousands at most. Since the minimum lethal dose of the latter proved to be at least 0.001 cc., the virulence of the organisms had been increased at least many thousand times by two successive passages in guinea pigs.

The low virulence of the initial culture shows that *Leptospira icteroides* as it occurs in human yellow fever is only slightly pathogenic for guinea pigs, an enormous number of organisms being required to bring about infection. In order to establish a strain virulent for guinea pigs it is necessary to transfer the passage strain during the febrile stage of the infection. Usually it is necessary also to enrich the patient's blood in culture medium before inoculating guinea pigs, and the use of suitable culture medium and of susceptible (young) animals in as large number as practicable is indispensable. Moreover, the larger the number of cases from which transmission is attempted, the greater the possibility of encountering a strain of moderate pathogenicity for guinea pigs. It was only after a third passage through the guinea pig that Noguchi in Guayaquil and Le Blanc in Vera Cruz succeeded in establishing a strain virulent for guinea pigs. It was inevitable, therefore, that the average transmission experiments made in the past should have failed, inasmuch as attempts were made to induce a fatal infection at once in guinea pigs by introducing the very limited number of leptospiras of an unadapted strain present in the peripheral blood of yellow fever patients.

Susceptibility of Certain Monkeys to Leptospira icteroides, Palmeiras Strains.

Macacus rhesus monkeys had proved resistant to the *icteroides* strains isolated from yellow fever cases in Guayaquil, while the Colombian

¹⁰ A parallel series of experiments in marmosets gave unsatisfactory results, owing to loss of the animals from other causes.

varieties of marmosets succumbed to the infection with pronounced symptoms and lesions.⁹ Following the isolation of the Palmeiras strains, there was opportunity to test their virulence for monkeys of three different varieties, two African baboons, taken from New York, and three Brazilian monkeys, two of the species *Cebus macrocephalus*, and one *Ateles ater*. The material used for infection was a mixture of rich cultures of the second passage strains, and the quantity was uniformly 1 cc. The inoculations were subcutaneous in all instances.

The baboons were inoculated first. Except for a rise of temperature for a few days, the animals remained entirely unaffected by the procedure.

On February 11, 1924, at the suggestion of Professor Pirajá da Silva, the two *Cebus* monkeys were inoculated. Both showed fever after 60 hours. The first febrile period persisted for about 12 hours, when there was a brief remission, followed by a rise to 105.8–106°F. on the 4th day; the temperature then rapidly fell. One of the animals, which had the higher temperature, was given 15 cc. of anti-icteroides immune horse serum intraperitoneally on the 4th day. No further symptoms developed, and the animal has since remained well. The untreated animal became gradually weak and listless and refused food (food taken during the early period of fever had been vomited). Later it fell to the floor of the cage and seemed delirious, offering slight resistance to handling. Coma developed subsequently, and death occurred 7 days after the first rise of temperature.

Autopsy.—Well developed rigor mortis; faint trace of generalized jaundice, scleræ and ear lobes more distinctly yellow. No hemorrhages in the subcutaneous or muscular tissues. Lungs almost normal in appearance but having a few minute punctate hemorrhages in the left lobes and a general pinkish yellow color; no adhesions or pleural exudate. Tongue coated, with free edges and tip; gums congested. Liver (Fig. 3) pale yellow, fatty, more yellowish in left lobe, which showed some necrotic areas (?). (Compare with normal liver of monkey of same species, shown in Fig. 4.) Spleen normal in size and color. Stomach (Fig. 5) contained a considerable amount of black or brownish viscid liquid adherent to the mucosa (black vomit). Clots in both auricles and ventricles of heart. Kidneys of normal size, capsule stripped easily; subcapsular hemorrhage; cut surface slightly yellow; some congestion in cortex, medulla grayish pink and congested, cortex slightly opaque. Adrenals normal in size and color. Bladder completely empty (anuria). Few hemorrhagic spots in small intestine, of which contents were dark greenish yellow, stained with bile and in some places with blood (melena).

Large intestine contained partly formed feces of tarry color and consistency. Dark-field examination of blood and organ suspensions negative for leptospira. Cultures of heart blood negative.

Histological Report (Dr. Muller).—Liver. In sections fixed in formalin and stained with hematoxylin and eosin, the liver trabeculae are distinct and the liver cells large, polyhedral, and vacuolated. The vacuoles are variable in size, and there are six to ten in the section of each cell. Because of the closely packed vacuoles, the cytoplasm is reticulated. The nuclei of the cells are large and round, and most of them lie towards the center of the cell. The blood sinuses are dilated and filled with blood, and the endothelial lining cells have prominent flat nuclei projecting into the sinuses. The portal canals (blood vessels and bile ducts) appear normal in structure.

In sections stained with scarlet R and counterstained with hematoxylin (Fig. 6), the fat globules fill almost the entire liver cell, and every lobule, except for a narrow zone around the central vein, is completely fatty. Towards the periphery of the lobules the fat globules tend to be larger and conglomerate. The epithelium of the bile ducts presents no fatty change, nor is there any fatty degeneration of the endothelium of the blood vessels in the sections studied.

No leptospiras were found in sections stained by Levaditi's silver impregnation method.

Kidney. In the hematoxylin and eosin sections the epithelial cells of the convoluted tubules and of the collecting tubules show no distinct cell outlines but appear as a red staining granular and vacuolated mass which nearly completely occludes the lumen. The nuclei are smaller than normal and pycnotic. Minute hemorrhages are present in the medulla of the kidney. The glomeruli are normal.

In sections stained with scarlet R and hematoxylin (Fig. 7) the epithelial cells of the convoluted and collecting tubules are the seat of numerous closely packed fine fat droplets, situated mainly at the base of the cell. The fat deposit is very marked throughout the epithelium of the tubules. The glomeruli present no fat, and the blood vessels and capillaries appear normal.

No leptospiras were encountered in sections impregnated with silver by Levaditi's method.

Adrenal. The histological appearance is normal. There is no hemorrhage or necrosis. Levaditi sections show no leptospira.

Heart. In the hematoxylin and eosin sections the striations are visible but slightly less distinct than normal. The fibers are the seat of numerous very minute vacuoles. The nuclei are normal, and there is no pigment at the poles of the nuclei. No hemorrhages are present, and no other form of degeneration (such as Zenker degeneration) can be found.

In the sections stained with scarlet R and hematoxylin (Fig. 8), numerous very fine fat droplets are sprinkled uniformly throughout the entire length of all the muscle fibers. There is no accumulation of fat around the nuclei.

Levaditi sections reveal no leptospira.

Spleen. There is no essential difference between the microscopic appearance of this spleen and that of a normal *Cebus macrocephalus* monkey, except a very slightly smaller amount of lymphoid tissue than in the normal control. Phagocytosis of red cells is not present.

Lungs. The alveoli are free from exudate, but the alveolar walls are thickened, particularly around bronchioles, owing to increase in the fixed tissue cells with oval nuclei and to infiltration of a few polynuclears and large and small mononuclears. The capillaries are moderately congested. There are some large cells in the thickened walls, which contain dark brown or black pigment granules.

Sections stained with scarlet R show no fatty degeneration of the endothelium of the capillaries. No leptospiras are found in sections stained by Levaditi's method.

Stomach. The mucosa shows extensive postmortem change; the nuclei and cytoplasm of the epithelium are indistinct, and the glands are separated from the basement membrane. There are defects in the mucosa extending part or all of the way to the muscularis mucosæ, filled with dark brown coarse and fine granules of blood pigment. The cells lining the edges of these defects contain pigment in the cytoplasm, and over the entire mucosa there is a faint brown tinge of the autolyzed cells along the surface. By Perl's Prussian blue method (Fig. 9) for demonstrating hemosiderin (derived from the hemoglobin of the red blood corpuscles), this pigment stains where it is within or in contact with the epithelium, hence there are numerous blue patches over the surface epithelium, and the mucosal defects have blue linings.

Scarlet R stain does not bring out any fatty degenerative changes.

Large intestine. Hematoxylin and eosin sections present nothing unusual, except postmortem autolysis of the surface of the epithelium.

The complete picture of yellow fever was thus strikingly reproduced in this species of monkey (*Cebus macrocephalus*), which lives in the interior of Brazil (Amazonas).

The black spider monkey (*Ateles ater*), also from Amazonas, was inoculated at the same time as the two *Cebus*. A temperature of 105°F. was noted on the afternoon of the 3rd day, but there was no other symptom, the animal's appetite remained good throughout the period of experiment, and no jaundice was noted at any time.

As controls, six guinea pigs were simultaneously inoculated with 0.2 cc. of the same culture. All developed typical symptoms and succumbed in 5 to 7 days, the majority in 6 days, after inoculation.

Virulence of the Palmeiras Strains for Young Dogs.

Early in the course of the first transmission experiments of Noguchi in Guayaquil,⁹ it was found that young dogs (6 weeks old) developed

typical and usually fatal yellow fever when inoculated with sufficiently large quantities of virulent material from guinea pigs infected with *Leptospira icteroides*. Later experiments on puppies with strains of *Leptospira icteroides* from Mexico and Peru yielded similar results.¹¹ The experiments to determine the pathogenicity of the Brazilian strains for young dogs were carried out at The Rockefeller Institute after the return of the International Health Board Commission from Brazil.

Two pups of a litter about 8 weeks old were inoculated on May 26, 1924, one intraperitoneally and the other subcutaneously, each with 5 cc. of a rich culture of *Leptospira icteroides*, Brazilian Strain 5. Temperatures taken for 3 days preceding inoculation had ranged between 100° and 102.5°F., and the highest temperature recorded during the week following the injection was not above 102.5°. On the following Monday morning, which was the 7th day after inoculation, one of the animals was found dead, the other was comatose. Both were deeply jaundiced. The second animal died in the afternoon of the same day.

Autopsy.—Both animals were deeply jaundiced, one slightly less than the other. The stomach in each instance contained “coffee-ground” material, but no food. There were petechial hemorrhages in the mucosa and hemorrhages between mucosa and muscularis, and hemorrhagic areas were found in the mucosa of small and large intestine. The livers were pale and mottled, with lighter colored patches. Hemorrhages into the myocardium were present in both hearts. The lungs of one dog contained a few small hemorrhages, in the other the lung hemorrhages were more numerous. The spleen in each instance was normal. The kidney of one animal was pale, and the bladder contained no urine; in the other the kidneys were large and extensively hemorrhagic, and there were only 2 or 3 drops of blood-tinged, highly albuminous (Heller’s test) urine in the bladder.

Microscopic sections of the livers stained with scarlet R and hematoxylin revealed hemorrhage and extensive fatty infiltration of the liver cells. In sections of the kidneys, similarly stained, there was fatty infiltration of the cells of the collecting tubules, and the kidneys which had shown extensive macroscopic hemorrhages also had fat in the glomerular cells, and in the walls of the smaller blood vessels. In this dog also fine droplets of fat were found in patches of heart muscle.

As in the case of the monkey, the leptospira was not demonstrable by dark-field examination in blood or organs taken at autopsy.

¹¹ Noguchi, H., *Lancet*, 1922, i, 1185.

In sections impregnated with silver by Levaditi's method, however, the liver and kidney of both pups showed the presence of a few fragmented leptospiras, and the organisms were more numerous in the muscularis of the gastrointestinal tract. The difficulty of finding the leptospira in autopsy materials is not peculiar to the experimental disease, but is true also of human yellow fever.

Filtration Experiments with Brazilian Strain 5.

That the various strains of *Leptospira icteroides* pass through the pores of Berkefeld filters had been previously demonstrated by Noguchi,¹² Nichols,¹³ and Dieterich.¹⁴ For determination of the filterability of the Brazilian strains, there was available a single subculture (second) from the initial culture of Palmeiras Case 5, many subcultures having been lost through mould contamination. It was 2 weeks old and contained numerous active leptospiras. On February 2, 1924, 1 cc. of the culture was diluted with 100 cc. of sterile saline solution and the mixture shaken to liberate the organisms from the semisolid medium. A few leptospiras were found in each field on dark-field examination. 5 cc. of a fresh broth culture of a minute motile bacillus were added to the diluted culture, and the whole was divided into three equal portions, each of which was filtered through a separate Berkefeld filter.

Portion 1 was first filtered through a Berkefeld V filter, but because of the possibility of contamination in handling, it was refiltered through a Berkefeld N. Each of three guinea pigs received 5 cc. of the filtrate intraperitoneally, and six tubes of leptospira medium were inoculated with 1 cc. each. Dark-field examination revealed no leptospira in the filtrate, but one tube of the six showed good growth 2 weeks later. Of the three guinea pigs, two died of pneumonia within 3 days, the other remained well.

Portion 2 was passed through a Berkefeld V filter, and the filtrate, which was negative on dark-field examination, was distributed into six culture tubes and inoculated into three guinea pigs. Five of the six tubes developed excellent growth (Drs. Godofredo Vianna and

¹² Noguchi, H., *J. Exp. Med.*, 1919, xxx, 13.

¹³ Nichols, personal communication.

¹⁴ Dieterich, F. H., *Am. J. Trop. Med.*, 1924 (in press).

Horacio Martins). One of the guinea pigs died shortly afterwards of pneumonia, the other two escaped infection.

The third portion was passed through a Berkefeld N filter, and two guinea pigs and six culture tubes were inoculated. Subsequent dark-field examination showed that the filtrate contained a small number of the bacilli and occasional leptospiras, and it was found that the filter had not been tightened sufficiently. The six culture tubes were all contaminated by the next morning. One guinea pig remained well, the other died of pneumonia in 3 days.

The number of positive cultures obtained from the first and second experiments, respectively, showed that some leptospiras pass through filters of the N grade and more through those of the V grade. The negative results of animal inoculations were due to the low pathogenicity of the culture, which had not been passed through guinea pigs. The pathogenicity of the cultures obtained from the filtrates was tested by pooling the upper portion (1 cc.) of each of the twelve cultures and inoculating 2 cc. of the mixture intraperitoneally into four guinea pigs (February 16, 1924). Two of these animals, Guinea Pig A, Cage 133, and Guinea Pig A, Cage 124, had definite fever and showed characteristic lung lesions when sacrificed for examination 7 days after inoculation. The others had similar but milder reactions. Further passage, which would undoubtedly have established the virulence of the strain for guinea pigs, was not regarded as necessary.

IMMUNOLOGICAL STUDIES.

The Pfeiffer Reaction.

A Pfeiffer reaction carried out in April, 1923,¹⁵ with the serum of an American who had had yellow fever in Fortaleza, Ceará, 65 days previously, had indicated that the etiological agent in this case of yellow fever in Brazil was identical with the strains of *Leptospira icteroides* isolated elsewhere. At the time when our work in Bahia was begun (November, 1923), no cases were available for isolation experiments, but an excellent opportunity was offered to test the immunological properties of the sera of persons recovered from yellow fever in relation to the strains of *Leptospira icteroides* obtained from cases of the disease in Ecuador,¹² Mexico,⁵ and Peru.⁴ Through the kind cooperation of the physicians who had treated the cases,¹⁶ we obtained nine sera from residents of Bahia, chiefly foreigners, who had had yellow fever during the year 1923.

The procedure consisted in adding 1 cc. of a rich suspension of *Leptospira icteroides* culture to 1 cc. of a given blood serum and injecting the mixture into the peritoneal cavity of a normal guinea pig. After 30 minutes the peritoneal fluid was withdrawn and examined under the dark-field microscope. The sera of four persons who had never had yellow fever were simultaneously tested as controls, and both yellow fever convalescent and normal sera were also tested with *Leptospira icterohæmorrhagiæ* in parallel series in order to determine the specificity of the *icteroides* as compared with the *icterohæmorrhagiæ*. The strains of *Leptospira icteroides* used were Guayaquil No. 1, Le Blanc,¹⁷ Peru No. 2, and Vera Cruz,¹⁸ and of *Leptospira icterohæmor-*

¹⁵ Noguchi, H., *Am. J. Trop. Med.*, 1924, iv, 131.

¹⁶ We are indebted for the specimens of sera to Drs. Fernando Luz, Alberto do Rio, Dias do Moraes, Vidal da Cunha, and Eduardo Araujo.

¹⁷ This strain was isolated by Dr. Thomas J. Le Blanc from cases of yellow fever in Vera Cruz, Mexico, in 1920.

¹⁸ Grovas, P. P., *J. Am. Med. Assn.*, 1921, lxxvi, 362.

rhagiæ Group 30,¹⁹ American No. 2,²⁰ and L-C.²¹ The experiments were conducted by us jointly on December 20, 1923, and were repeated on 2 successive days for the benefit of those who were unable to be

TABLE III.
Results of Pfeiffer Tests on Yellow Fever Convalescents in Bahia.

Serum.	Sex.	Age.	Nationality.	Period elapsed since recovery.	Pfeiffer reaction.	
					<i>Leptospira icteroides.</i>	<i>Leptospira ictero-hæmorrhagiæ.</i>
				mos.		
F.....	M.	Adult.	American.	6	+	—
St.....	"	"	German.	6	+	—
Sal.....	"	"	Arabian.	9	+	—
Le.....	"	"	British.	10	+	—
Ley.....	F.	"	German.	7	+	—
B.....	M.	"	British.	7	+	—
So.....	"	"	Spanish.	5	+	—
M.....	"	"	Brazilian.	6	+	—
San.....	"	"	Portuguese.	9	+	—
Normal serum No. 1.....			Brazilian.		—	—
" " " 2.....			"		—	—
" " " 3.....			"		—	—
" " " 4.....			"		—	—
Saline solution					—	—

present on the 1st day of the demonstration.²² The results, which are recorded in Table III, clearly demonstrate the presence of a specific immune substance in the sera of yellow fever convalescents and

¹⁹ Noguchi, H., *J. Exp. Med.*, 1919, xxx, 95.
²⁰ Noguchi, H., *J. Exp. Med.*, 1917, xxv, 755.
²¹ Wadsworth, A., Langworthy, H. V., Stewart, F. C., Moore, A. C., and Coleman, M. B., *J. Am. Med. Assn.*, 1922, lxxviii, 1120.
²² The tests were conducted by Drs. Octavio Torres, Flaviano Silva, Horacio Martins, Alvaro Ribeiro dos Santos, Muller, and Macintosh. The following representatives of the medical profession in Bahia were present at one or more of the demonstrations: Professors Augusto Vianna, Gonçalo Moniz, Pirajá da Silva, João Fróes, Prado Valladares, Mario Andréa, Agrippino Barbosa, and Drs. Sebastião Barroso, Scannell, Didier, Dionysio Pereira, Vianna Junior, and Genesio Salles.

its absence from normal human sera, also the absence of the immune reaction when the sera were tested against *Leptospira ictero-hæmorrhagiæ*.

Following the isolation of two strains of *Leptospira icteroides* from cases of yellow fever in Palmeiras, Pfeiffer tests were made with these strains (January 20, 1924) and the convalescent sera from Bahia cases, also with positive results. Hence the identity of the disease occurring in Palmeiras and Bahia was established.

TABLE IV.

Results of Pfeiffer Tests on Yellow Fever Convalescents in Palmeiras.

Serum.	Sex.	Age.	Period elapsed since onset.	Pfeiffer reaction.	
				<i>Leptospira icteroides.</i>	<i>Leptospira ictero-hæmorrhagiæ.</i>
		yrs.	days		
Case 3, A. V. B.....	F.	23	28	+	—
“ 4, J. V.....	M.	18	31	+	—
“ 5, J. B. d. S.....	“	25	28	+	—
“ 7, B. d. A.....	F.	22	12	+	—
Normal serum control.....				—	—
Saline control.....				—	—

On February 1, 1924, Dr. Bião returned from Palmeiras and brought with him specimens of serum from four persons (Cases 3, 4, 5, and 7) who had had yellow fever 2 to 6 weeks previously. Cases 3 and 5 were those from which the strains of *Leptospira icteroides* had been isolated. These sera were tested²³ with the Palmerias strains as well as with those from Guayaquil, Peru, and Mexico, with positive results in all instances (Table IV). On a later occasion (February 21, 1924), similar tests were made²⁴ of serum taken on the 14th day of illness from

²³ The tests were carried out by Drs. Godofredo Vianna, Mario Bião, Horacio Martins, and Vianna Junior.

²⁴ The tests were made by Drs. Muller, Flaviano Silva, Alvaro Ribeiro dos Santos, Horacio Martins, Godofredo Vianna, and Mario Bião.

a clinically clear case of yellow fever in Conceição de Almeida, a town 2 days journey from Bahia. The results were the same as those obtained with the sera from Bahia and Palmeiras cases. The conclusion is warranted, therefore, that these yellow fever cases in Brazil were etiologically identical with those occurring in Ecuador, Peru, Mexico, and Colombia.¹⁵

A detailed report, with complete clinical history of each convalescent whose serum was tested, will be made in a forthcoming publication by Dr. Octavio Torres.

TABLE V.

Potency of Anti-icteroides Immune Serum against the Brazilian Strains of Leptospira icteroides.

Each animal received 1,000 M.L.D. (1 cc.) of Palmeiras Case 3 strain, Jan. 16, 1924.

Guinea pig No.	Serum.	Results.
	cc.	
107 A	1	Completely protected.
107 B	1	" "
108 A	0.1	" "
108 B	0.1	" "
109 A	0.01	" "
109 B	0.01	" "
110 A	0.001	Died of intercurrent infection (pneumonia) in 6 days. No sign of <i>icteroides</i> infection.
110 B	0.001	Died of intercurrent infection.
111 A	0.0001	" overnight of pneumonia.
111 B	0.0001	Completely protected.

Determination of Potency of Anti-icteroides Immune Serum against the Palmeiras Strains of Leptospira icteroides.

The anti-*icteroides* immune serum is that derived from horses which have been under immunization for several years with live cultures of strains of *Leptospira icteroides* from sources in Ecuador, Peru, and Mexico. It has been used therapeutically in various South and Central American countries.²⁵ It has also been tried in Brazil with variable

²⁵ Noguchi, H., *J. Am. Med. Assn.*, 1921, lxxvii, 181.

results.²⁶ It was of interest, therefore, to determine experimentally the minimum quantity of the serum which would protect guinea pigs against a given number of minimum lethal doses of the Brazilian strains of the *icteroides*, when administered simultaneously. The particular lot of serum used had been obtained at a bleeding 2 months previous to the experiment and had been kept at 4°C. during its transportation to Bahia and at 12°C. after its arrival there.

A virulent suspension of blood and kidney of Palmeiras Case 3 strain was employed for infection. Titration of the M.L.D. of this material, made simultaneously with the titration of the serum, showed that 0.001 cc. killed guinea pigs in 8 days. 1 cc. of the suspension was injected intraperitoneally, and the animals received varying doses of the anti-*icteroides* serum within 30 minutes. Two guinea pigs were used for each dose of the serum (Table V).

Although three animals were lost through intercurrent infection, none of the others showed fever or any other symptom of the *icteroides* infection. The minimum protective quantity of the serum against 1,000 M.L.D. was at least 0.0001 cc.; that is, 1 cc. of the serum was able to neutralize at least 10,000,000 M.L.D.

Experiments on the treatment of infected animals with the serum at different stages of the disease had to be postponed because the supply of guinea pigs brought from New York was exhausted. A small series of experiments was attempted with native guinea pigs of various sizes (100 to 300 or 400 gm.), but, as was expected, consistent results were not obtained, many controls escaping infection, while the few very young animals which alone became infected proved to be too delicate for serotherapeutic experiments.

²⁶ Barreto, A. L. B., *A Folha Med.*, 1920, i, 152. Cavalcanti, E. P., These, Faculdade de Medicina do Rio de Janeiro, 1921.

SUMMARY.

Two strains of *Leptospira icteroides* have been isolated from two of nine cases of yellow fever in Villa Bella das Palmeiras, an interior town in the state of Bahia, Brazil, by inoculation of suitable culture medium with blood drawn on the 1st and 2nd days of illness, respectively.

The characteristic pathogenicity of the Brazilian strains of *Leptospira icteroides* was established by experiments in guinea pigs taken from New York. The original cultures, directly isolated from the blood of patients, showed a very low virulence for guinea pigs, but by timely passage to fresh animals during the height of fever, the virulence of the strains had been increased several thousandfold in the third generation. The essential features of the infection were jaundice, hemorrhages into the lungs and gastrointestinal mucosa, nephritis, and fatty degeneration of the liver. The leptospira was rarely demonstrable in the materials used for transmission, and the success of cultivation was variable and never readily accomplished.

Two monkeys of the species *Cebus macrocephalus*, when inoculated with the Brazilian strains of the second passage, developed typical symptoms of severe yellow fever. One recovered after having received anti-*icteroides* immune serum (horse) on the 4th day of illness; the other died on the 7th day. Autopsy revealed the pathological changes typical of human yellow fever, and histological study of the organs demonstrated the presence of the characteristic severe fatty degenerative changes of liver and kidney. Three African baboons and a monkey of the species *Ateles ater*, similarly inoculated, developed slight fever on the 3rd or 4th day, but otherwise remained well.

The Brazilian strains of *Leptospira icteroides* induced in young dogs a fatal infection, characterized by jaundice, hemorrhages (principally into the gastrointestinal tract, with black vomit), and intense nephritis. Fatty degeneration of liver and kidney was marked. Dark-field examination and cultures were negative, but the leptospira was demonstrated in tissues stained by Levaditi's method.

The filterability of the Brazilian strains was established by the recovery of the leptospira in culture medium inoculated with Berkefeld V and N filtrates of the original culture of Strain 5. The cultures obtained from the filtrates had the same degree of pathogenicity for guinea pigs as the initial culture.

Sera from nine persons who had had yellow fever in Bahia 5 to 10 months previously, four sera from Palmeiras cases 2 to 6 weeks after their attack of the disease, and one serum taken on the 14th day of illness from a yellow fever patient in Conceição de Almeida all gave positive Pfeiffer reactions when tested with strains of *Leptospira icteroides* from sources in Ecuador, Mexico, and Peru, as well as with the Brazilian strains. Parallel reactions with *Leptospira icterohæmorrhagiæ* were uniformly negative. Several normal sera tested in each instance as controls gave negative reactions with both organisms. The identity of the yellow fever occurring in Ecuador, Mexico, Peru, Colombia, and Brazil was thus established.

0.0001 cc. of the anti-*icteroides* serum prepared in horses with strains of *Leptospira icteroides* of Ecuadorean, Mexican, and Peruvian origin protected guinea pigs against 1,000 M.L.D. of a Brazilian strain.

EXPLANATION OF PLATES.

PLATE 1.

Dark-field views of the strains of *Leptospira icteroides* isolated from cases of yellow fever in Palmeiras. Magnification, $\times 3,000$.

FIG. 1. Culture of Palmeiras Strain 3.

FIG. 2. Culture of Palmeiras Strain 5.

PLATE 2.

FIG. 3. Gross appearance of liver from *Cebus macrocephalus* monkey inoculated with Brazilian strains of *Leptospira icteroides*. Natural size.

FIG. 4. Liver of normal *Cebus macrocephalus* monkey (for comparison with Fig. 3). Natural size.

FIG. 5. Inner surface of stomach of *Cebus macrocephalus* monkey inoculated with the Brazilian strains of *Leptospira icteroides*, showing the black liquid adherent to the mucosa (black vomit). Natural size.

PLATE 3.

FIG. 6. Section of liver of *Cebus macrocephalus* monkey inoculated with the Brazilian strains of *Leptospira icteroides*, showing severe grade of fatty degeneration. Stained with scarlet R and hematoxylin. Magnification, $\times 92$.

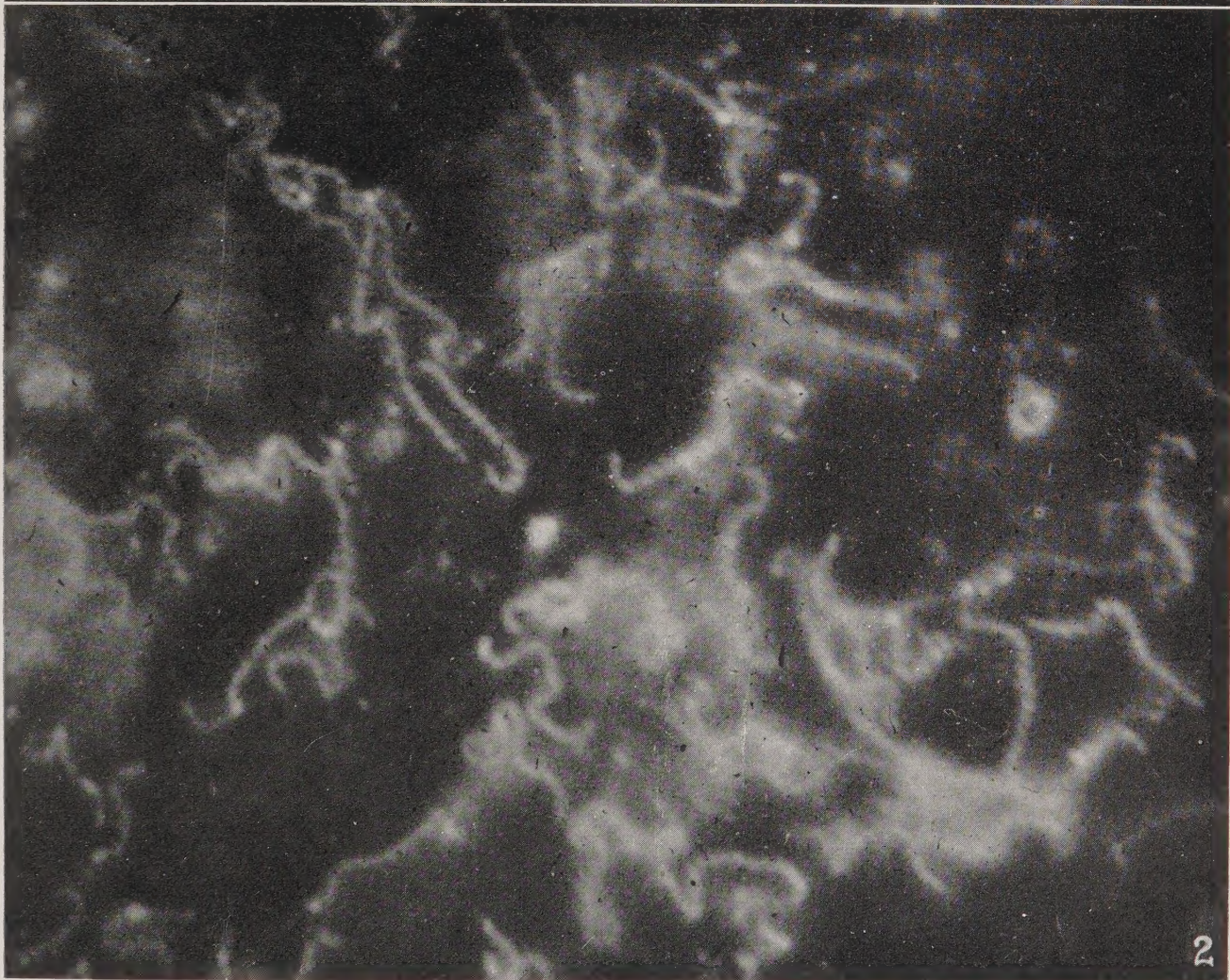
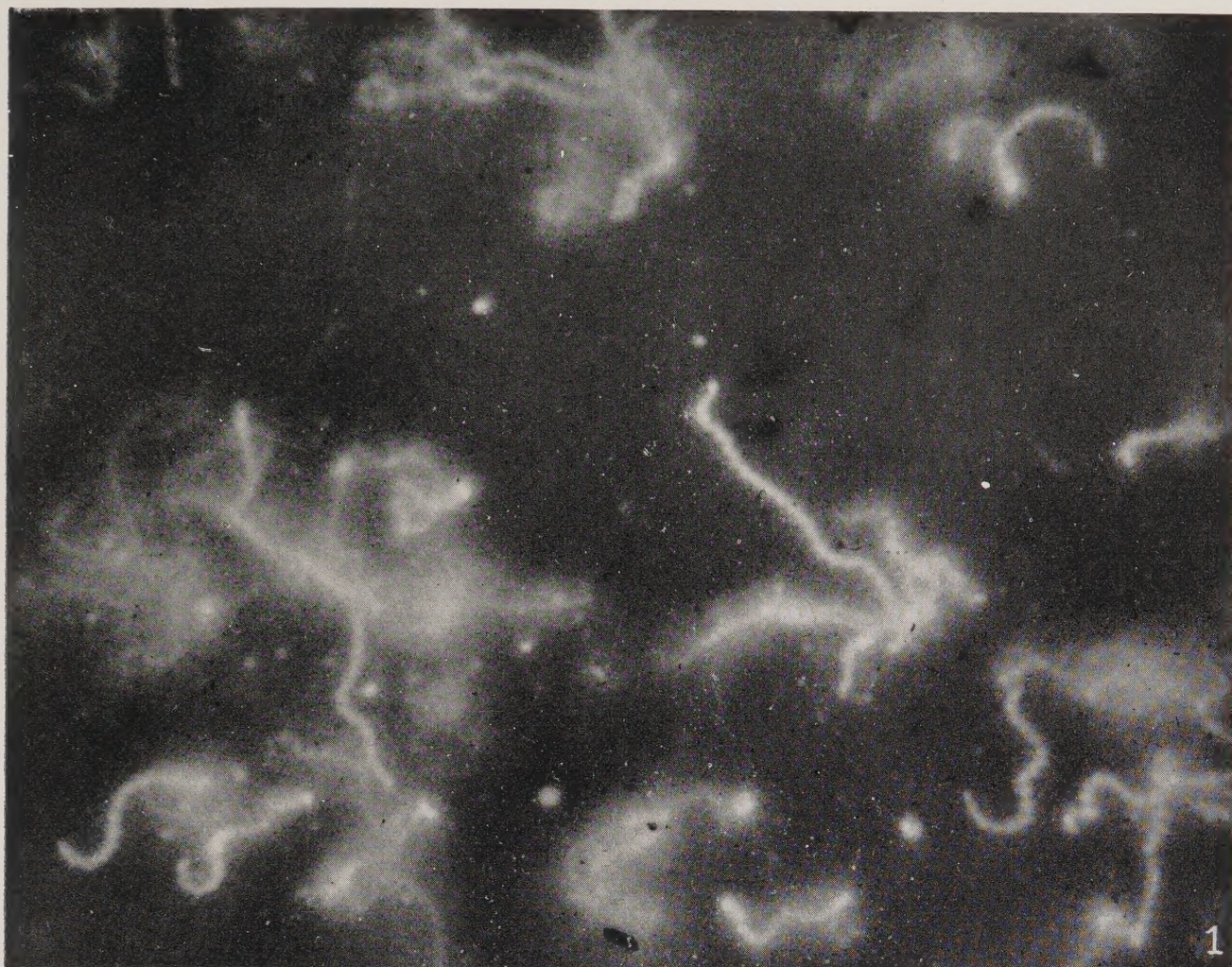
PLATE 4.

FIG. 7. Section of kidney of *Cebus macrocephalus* monkey inoculated with the Brazilian strains of *Leptospira icteroides*, showing fatty degeneration of epithelium of tubules. Stained with scarlet R and hematoxylin. Magnification, $\times 30$. In the lower left-hand corner is a higher magnification ($\times 200$) of the same section. Glomerulus unaffected.

PLATE 5.

FIG. 8. Section of heart muscle from *Cebus macrocephalus* monkey inoculated with the Brazilian strains of *Leptospira icteroides*, showing uniform fatty degeneration of all the muscle fibers. Stained with scarlet R and hematoxylin. Magnification, $\times 400$.

FIG. 9. Section of wall of stomach of the same animal, showing altered blood pigment (hemosiderin) adherent to the mucosa. Stained by Perl's Prussian blue method. Magnification, $\times 30$.



(Noguchi *et al.*: Yellow fever in northern Brazil.)



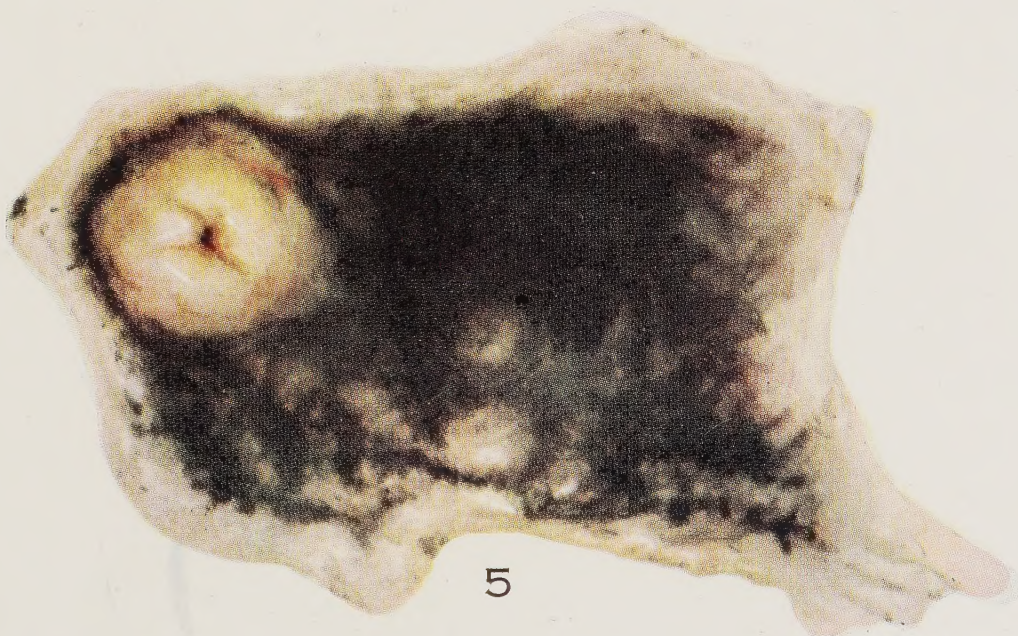
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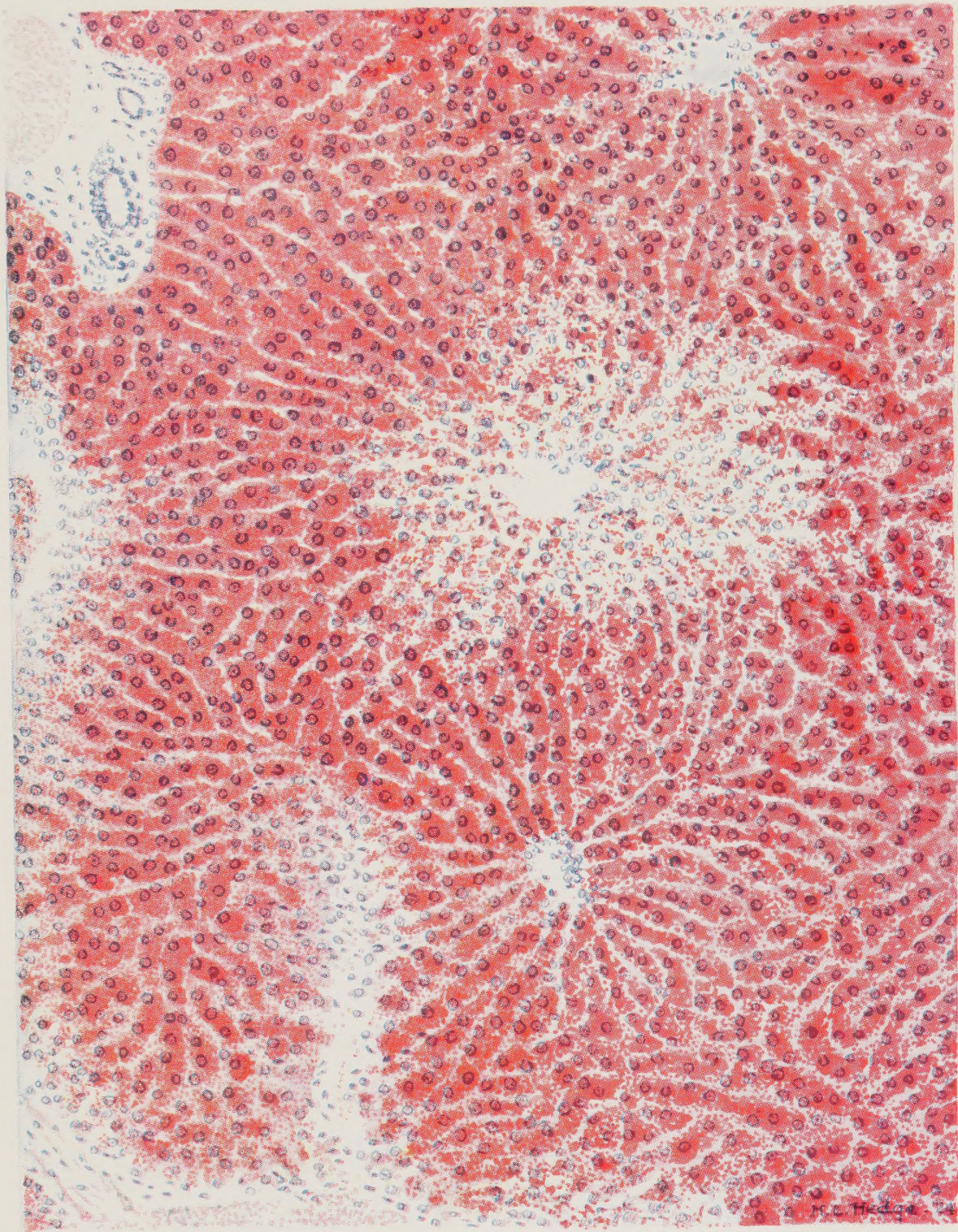
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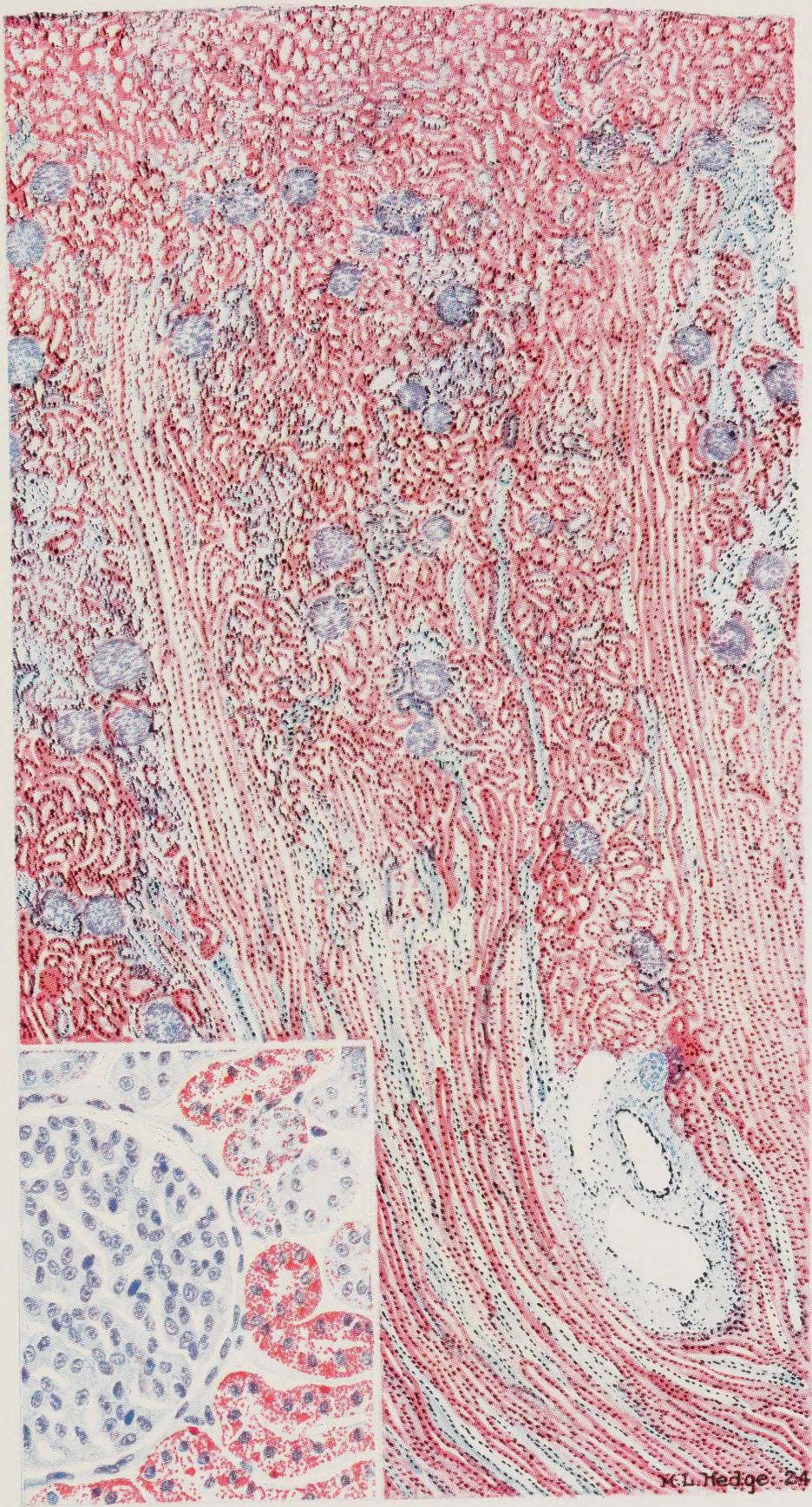


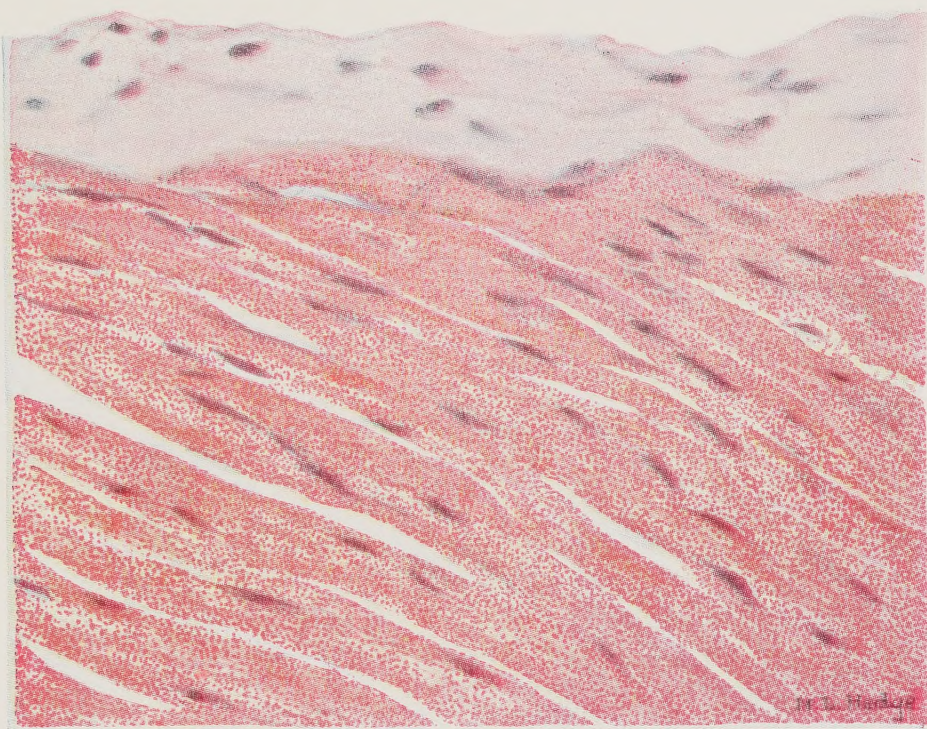
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